ABNORMALLY HIGH RATE OF CYCLIC AMP EXCRETION FROM AN ESCHERICHIA COLI MUTANT DEFICIENT IN CYCLIC AMP RECEPTOR PROTEIN

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SUMMARY

By labeling adenosine 3', 5'-cyclic monophosphate (cyclic AMP) with [^{32}P] phosphate and chromatographing it on a thin-layer alumina plate, we have determined the extra- and intracellular amounts of cyclic AMP in an Escherichia coli CRP$^-$ mutant (deficient in a cyclic AMP receptor protein) and its isogenic CRP$^+$ cell. The CRP$^-$ cell was found to excrete cyclic AMP at an abnormally high rate as compared to the CRP$^+$ cell when growing on glucose or glycerol, which can be correlated with the abnormally high intracellular levels of cyclic AMP in the CRP$^-$ cell.

Adenosine 3', 5'-cyclic monophosphate (cyclic AMP) is required for the synthesis of β-galactosidase and other inducible catabolic enzymes in Escherichia coli (1). This action of cyclic AMP requires its interaction with a specific protein, referred to as the cyclic AMP receptor protein (CRP) (2). CRP is assumed to be identical with the catabolite gene activator protein (CAP) (3). An E. coli CRP$^-$ mutant (deficient in functional CRP) can not grow efficiently on carbon sources whose utilization requires the induction of catabolic enzymes (2). The cyclic AMP-CRP control acts on the transcription of the operons of these inducible enzymes (4-6) presumably via a specific interaction with the promoter regions of the operons (7, 8). However, the exact mode of action of CRP is not yet understood.

The present communication reports that an E. coli CRP$^-$ mutant excretes cyclic AMP at an abnormally high rate.

MATERIALS AND METHODS

Bacteria and Culture Conditions: Three E. coli K-12 strains were used: 1100 (crp$^+$, thi$^-$), 5333 (crp$^-$, thi$^-$), and R5333. 1100 and 5333 (2) were provided by Dr. Ira Pastan, National Cancer Institute, Bethesda, Md. A spontaneous CRP$^+$ revertant (designated R5333) was isolated from 5333 by us. We have confirmed that 5333 can not ferment lactose and maltose on MacConkey and tetrazolium plates. Galactose and arabinose (both from Sigma) were slightly fermented by 5333 on these plates. Isopropyl-thio-β-D-galactoside (IPTG) did not induce β-galactosidase in 5333 with or without exogenous cyclic AMP. Unlike an adenyl cyclase deficient mutant (9), 5333 was found to grow on glycerol (Baker No. 2136 or Fisher G-33) without exhibiting a long lag. Both 1100 and R5333 fermented efficiently arabinose, galactose, lactose and maltose as well as glucose and glycerol. Bacteria were grown by shaking at 37^o in Tris-maleate minimal medium (TMM) (10) supplemented with thiamine (10 µg/ml) and glucose (4 mg/ml), glycerol (4 mg/ml), or sodium pyruvate (5 mg/ml). Phosphate concentration in the medium was adjusted to 2×10^{-4} M. Cell mass density was measured by absorbance at 500 nm (A_{500}) with a Bausch and Lomb Spectronic 20 spectrophotometer using a round cuvette (11 mm inside diameter). A culture grown on glucose or glycerol at $A_{500} = 1.0$ corresponded to approximately 10^9 cells/ml.

Assay of Intracellular Cyclic AMP: Carrier-free [^{32}P]orthophosphate was added to an exponentially growing culture to final concentrations of 200 - 400 µCi/ml (No significant radiation effect on growth was observed for at least two doublings). After one doubling, duplicate samples (1.0 ml) were filtered through a Millipore filter (25 mm diameter) which had been soaked in unlabeled cyclic AMP (10^{-5}M) in TMM. The cells were quickly washed with three portions of 0.7 ml of TMM (the necessity of this step will be

reported elsewhere). The washed cells were immediately suspended in 0.2 ml of 1 M formic acid. The suspension (with the filter) was frozen and thawed 3 times to facilitate the extraction of cyclic AMP. The extract was then centrifuged for 5 min in a Beckman Microfuge. Cyclic AMP was chromatographed by the modified procedure of Potter and Yamazaki (11) which permitted the complete separation of cyclic AMP from nucleoside mono-, di-, or triphosphates and 2', 3'-cyclic or other 3', 5'-cyclic monophosphates. Twenty microliters of the supernate of the extract and 5 µl of authentic cyclic AMP (5 mM) were applied onto an alumina thin-layer precoated sheet (Eastman Kodak Co. No. 6062). The chromatogram was developed ascendingly for approximately 5 hours in a slanted chamber with a tight cover in the solvent: 1 M ammonium acetate (pH unadjusted) and ethanol (7:13, v/v). The air-dried chromatogram was developed in the second dimension with the same solvent. Cyclic AMP was located by both autoradiography and ultraviolet detection of unlabeled cyclic AMP added as a marker, and it appeared as a kidney-shaped spot well resolved from other phosphorylated compounds. The regions of the chromatograms corresponding to cyclic AMP were cut out, immersed into 15 ml of toluene-based scintillation fluid and counted in a liquid scintillation spectrometer. Known amounts of $[^{3}H]$cyclic AMP applied to the origin as controls were quantitatively recovered with the marker.

Assay of Extracellular Cyclic AMP: The cultures were labeled with $[^{32}P]$orthophosphate as described in the preceding assay. 0.2-ml portions of the cultures were withdrawn at various times and suction-filtered through a Millipore filter (10 mm diameter) on a Swinnex-13 filter unit which was mounted on a test tube with a side arm. Ten microliters of the filtrates were chromatographed as described in the preceding assay (in most cases, one-dimensional development alone was found to be sufficient for this assay)

Calculation of "Differential Rate": Since the rates of excretion of cyclic AMP from bacteria growing at different rates are to be compared, the differential rate plot introduced by Paigen (12) was employed. The molar concentrations of extracellular cyclic AMP at any time divided by the initial cell mass density (at the time of addition of $[^{32}P]$orthophosphate) were plotted against the relative mass increase (the mass density at that time divided by the initial mass density). The slope in a linear region of this plot (Fig. 1) is defined as the differential rate (Table I) or rate (in the text).

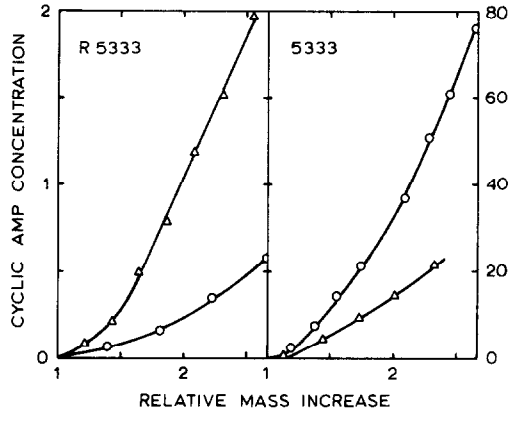

Fig. 1. Extracellular concentrations of cyclic AMP in strains R5333 (CRP^+) and 5333 (CRP^-). $[^{32}P]$Orthophosphate was added to exponentially growing cultures (at the relative mass increase = 1.0). Samples were withdrawn periodically and assayed for extracellular cyclic AMP as described under Materials and Methods. The ordinate indicates 10^7X cyclic AMP concentration (M) divided by A_{500} of the cultures at the time of addition of $[^{32}P]$orthophosphate (initial A_{500}). The abscissa indicates A_{500} (at the sampling time) divided by initial A_{500}. Symbols: O , cultures grown on glucose; Δ , cultures grown on glycerol.

RESULTS AND DISCUSSION

We have initially found that a CRP^- mutant 5333 excretes cyclic AMP at an abnormally high rate - at least 70 times greater than did its parent CRP^+ strain 1100 (Table 1). However, since 5333 has been

TABLE I

Comparison of Strains R5333 (CRP^+) and 5333 (CRP^-)

Characteristics	Carbon Source	R5333 (a)	5333
Doubling time in minutes	Glucose	65 (60)	110-115
	Glycerol	85	100-110
	Pyruvate	100-105	--
Differential rate of cyclic AMP excretion (b)	Glucose	0.70 (1.0)	72
	Glycerol	1.8	29
	Pyruvate	0.25	--
Intracellular cyclic AMP level (c)	Glucose	1.5, 1.4 (1.0, 1.0)	12, 12
	Glycerol	2.8, 2.9	7.0, 7.0
	Pyruvate	1.7, 1.7	--
ATP level (d)	Glucose	7.4, 7.6	4.6, 4.8

(a) The values in the parentheses are those of strain 1100 (CRP^+).

(b) 10^7X extracellular cyclic AMP concentration (M) / initial A_{500} / relative mass increase.

(c) Expressed as picomoles of intracellular cyclic AMP in 1.0 ml of culture at A_{500} = 1.0. The results of duplicate samples are presented.

(d) Expressed as nanomoles of ATP in 1.0 ml of culture at A_{500} = 1.0. The nucleotides were labeled with $[^{32}P]$orthophosphate and extracted with formic acid as described under Materials and Methods. $[^{32}P]$ATP separated by means of two-dimensional thin-layer chromatography (14) was counted. The results of duplicate samples are presented.

derived by treatment with N-methyl-N'-nitro-N-nitrosoguanidine (2) and this potent mutagen frequently causes multiple mutation (13), it is possible that the abnormal excretion of cyclic AMP is due to a mutation other than the crp mutation in 5333. If this were the case, a CRP^+ revertant from 5333 would still retain a capacity to excrete cyclic AMP at an abnormally high rate. A spontaneous Lac^+ stable revertant (designated R5333) was isolated from 5333 on MacConkey plate containing lactose. The revertant exhibited a similar dif-

ferential rate of β-galactosidase synthesis on addition of IPTG as 1100 did. Since the revertant fermented arabinose, galactose, and maltose as well, it must be a CRP^+ revertant but not a lactose promoter mutant (Lac^+) which is independent of the cyclic AMP - CRP control (8). Fig. 1 illustrates that the reversion to CRP^+ resulted in a marked reduction in the excretion rate of cyclic AMP: R5333 (CRP^+) excreted cyclic AMP at an approximately 1/100th the rate in 5333 (CRP^-) when growing on glucose. In glycerol medium, R5333 exhibited the excretion rate 1/16th that of 5333. The excretion rate in R5333 was greater in glycerol than in glucose, whereas the rate in 5333 was less in glycerol than in glucose. Table I summarizes several characteristics of this CRP^+/CRP^- pair. Since 5333 grows considerably slower than R5333 and 1100, one can suggest that the abnormal cyclic AMP excretion in 5333 is a consequence of slow growth rather than the crp mutation itself. However, such a possibility seems unlikely since R5333 slowly growing in pyruvate excreted cyclic AMP at a reduced (rather than increased) rate as compared to R5333 growing on glucose. It can be concluded that the abnormal excretion of cyclic AMP in 5333 is a direct consequence of the crp mutation. The higher cyclic AMP excretion rates in 5333 are correlatable with its abnormally high intracellular cyclic AMP levels in both glucose and glycerol (Table I).

The present results lead to interesting speculations on the functions of CRP: (I) CRP may be a major user of cyclic AMP. A CRP^- mutant can not utilize cyclic AMP formed, resulting in the abnormally high intracellular level and excretion rate of cyclic AMP. Then, why does a CRP^+ cell utilize cyclic AMP when the induction of catabolic enzymes is not required? Does CRP have other cellular roles? (II) CRP may directly regulate the synthesis or degradation of cyclic AMP affecting its intracellular level and excretion rate.

These possibilities are being examined by determining the synthetic and degradative rates of this nucleotide *in vivo* in a CRP^+/ CRP^- pair.

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